Early onset may predict G101W CDKN2A founder mutation carrier status in Ligurian melanoma patients

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Although the presence of multiple cases of melanoma on the same side of a family is the best predictor of germline CDKN2A mutation, other features (i.e. early age at onset) may be useful to identify carriers. We analysed the records of 682 hospital-based Ligurian melanoma patients. Of these, 238 cases (34 familial, 14 non-familial multiple primary and 190 non-familial single primary melanomas) were consecutively enrolled for screening of the CDKN2A and CDK4 genes. Screening of the 34 familial patients revealed that nine were carriers of the CDKN2A G101W founder mutation. Of the 14 non-familial multiple primary melanoma patients, three carried the G101W founder mutation and one the P48T mutation. For the non-familial patients with a single melanoma, 17 of 190 carried germline CDKN2A mutations, with most (16/17) carrying the G101W Ligurian founder mutation and one a novel single base pair substitution, D74Y. The effect of mutation on age at diagnosis was significant (*P*=0.012) after correcting for melanoma type (familial or non-familial), number of primaries (single or multiple), gender and disease occurrence (incident or prevalent). Early age at onset may be a good predictor of CDKN2A mutation in Liguria, where the G101W founder mutation is prevalent among melanoma patients, independent of family history. *Melanoma Res* 14:443–448 © 2004 Lippincott Williams & Wilkins.

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Introduction

Germline mutations in the CDKN2A and CDK4 genes have been most commonly detected in large, dense melanoma kindreds [1], and the presence of multiple cases of melanoma on the same side of a family is considered to be the strongest predictor of mutation [2]. Our previous study on 62 families from northern Italy [3], however, found that >30% of families carried the CDKN2A G101W mutation, mostly including kindreds with just two affected members. We have previously reported that the G101W mutation results from a single ancient event [4], and is prevalent amongst Ligurian melanoma patients [3].

In general, familial melanoma cases appear to have an earlier age at diagnosis than non-familial cases [5], and the incidence of CDKN2A mutations is higher in families with an early age at onset [2,6]. Moreover, the first tumour in multiple melanoma patients tends to develop earlier than in patients with a single melanoma [7].

With the aim of clarifying whether an early age at onset of melanoma, for example onset of the disease at ≤40 years, as suggested by other authors [8], is a good predictor of CDKN2A mutation in a population in which a founder mutation is prevalent, we studied a hospital-based series of Ligurian melanoma patients.

Methods

We reviewed retrospectively the clinical records of a hospital-based series of 682 melanoma patients who were consecutively seen between 1 January 1995 and 31 December 1999 at three hospitals in Liguria. Both incident (newly diagnosed) and prevalent (attending follow-up) patients were eligible, provided that they had

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primary, histologically confirmed diagnoses, including *in situ* melanoma (3%). The total number of eligible patients in the database was 667 of the 682: two were removed for a lack of diagnosis of the primary lesion and 13 for incomplete records (no histological type, no Clark and Breslow parameters). Of the eligible patients, 412 were incident and 255 were prevalent. Comparison with the Genoa Cancer Registry [9] showed that our sample covered 40% of the incident melanoma cases in 1995–1996.

Review of residence and survival data showed that, of the 667 eligible cases, 76 had moved and 48 had died before January 2000. Of the remaining pool of 543 patients, 238 agreed to participate in our study when approached during follow-up between January 2000 and June 2001, and 305 either declined or were not asked.

Patients were defined as familial if they had at least one first-degree relative with melanoma and as clinically sporadic (hereafter referred to as 'non-familial') if no family history of the disease, in relatives of any degree, was reported.

The set of 238 participating patients included 30 familial, 12 non-familial multiple primary melanoma (MPM) and 196 non-familial single primary melanoma (SPM) patients. At interview, six of the 196 non-familial SPM patients were reclassified for having an affected relative who had not been recorded ($n = 4$) or having a second melanoma ($n = 2$). Hence, 190 non-familial SPM, 34 familial and 14 non-familial MPM cases were recruited for DNA testing. After receiving detailed information on the study and providing informed consent, patients were tested for mutations in the CDKN2A and CDK4 genes.

This study was approved by the local National Cancer Institute (IST) Ethics Committee.

Information on participating and non-participating subjects, as recorded in the database, was compared, and it was found that they were not different with regard to gender, occurrence of the disease (incident or prevalent) and number of primaries (single or multiple). The subset of consenting patients, however, displayed a significantly lower median age at diagnosis (45.5 years vs. 55 years, $P < 0.001$) and included a significantly higher number of familial cases (30/238 vs. 10/305, $P < 0.001$).

Following DNA testing, all of the non-familial SPM mutation-positive cases were approached for a further interview to gather more information on family history.

CDKN2A and CDK4 gene analysis

Primer sequences and polymerase chain reaction (PCR) and sequencing conditions have been described previously [3].

Haplotype. analysis

Haplotypes were determined for 16 non-familial SPM G101W carriers, three G101W-positive non-familial MPM cases and nine familial G101W carriers with the following eight markers: IFNA, D9S736, D9S1749, D9S974, D9S942, D9S1748, D9S1604 and D9S171. Primer sequences and genotyping conditions have been described previously [10]. Six control DNAs with known allele size, provided by Dr Alisa Goldstein, were analysed for each marker.

Statistical analyses

The non-parametric Wilcoxon test was used to compare differences between age at diagnosis in the different groups. An analysis of variance (ANOVA) model correcting for melanoma type (familial or non-familial), number of primaries (single or multiple), gender and disease occurrence (incident or prevalent) was used to assess the impact of mutation on age at diagnosis (logarithmically transformed). Chi-square analyses (Yate's correction) and Fisher's exact test were used to compare differences in frequency distributions. Results were considered to be significant if the P value was < 0.05.

Results

Age at diagnosis

The distribution of age at diagnosis in the 238 cases (34 familial, 190 non-familial SPM and 14 non-familial MPM) recruited for screening of the CDKN2A and CDK4 genes is reported in Table 1. No significant differences in the age at diagnosis were detected between genders for all the melanoma groups; in non-familial SPM melanoma cases, the median age at diagnosis was 47 years for males and 45 years for females ($P = 0.71$), in familial cases it was 39 years for males and 46.5 years for females ($P = 0.34$), and in non-familial MPM cases it was 46.5 years for males and 59.5 years for females ($P = 0.57$).

Table 1 Distribution of age at diagnosis in melanoma patients

	Age at diagnosis (years)				
Melanoma group	16–30	31–50	51–70	71 +	Total
Familial	9 (26.5%)	12 (35.3%)	13 (38.2%)		34 (100.0%)
Non-familial SPM	35 (18.4%)	70 (36.8%)	66 (34.7%)	19 (10.0%)	190 (100.0%)
Non-familial MPM	3 (21.4%)	4 (28.6%)	6 (42.9%)	1 (7.1%)	14 (100.0%)
Total	47 (19.7%)	86 (36.1%)	85 (35.7%)	20 (8.4%)	238 (100.0%)

MPM, multiple primary melanoma; SPM, single primary melanoma.

Mutational analysis

Screening of the 34 familial patients revealed that nine were carriers of the G101W founder mutation. Of the 14 non-familial MPM patients, three carried the G101W founder mutation and one the P48T mutation. For non-familial SPM patients, 17 of 190 carried germline CDKN2A mutations, with most (16/17) carrying the G101W Ligurian founder mutation and one a novel single base pair substitution, D74Y. Overall, the frequency of CDKN2A mutation (99.2% accounted for by the G101W founder mutation) in the 238 patients tested was 12.6% ($n = 30$: nine familial cases, four non-familial MPM and 17 non-familial SPM). The CDKN2A 5′-UTR variant at position –34 [11] was found in none of the patients tested, and no mutation was found in CDK4 exon 2A (data not shown). No germline CDKN2A mutations were detected in 158 healthy geographic controls with no personal or familial melanoma history.

Age at diagnosis stratified by mutational status

The mean and median ages at diagnosis for mutation-positive and mutation-negative cases for each melanoma group are reported in Table 2. Interestingly, the median age at diagnosis of the 17 mutation-positive non-familial SPM melanoma cases was 32 years, compared with 46 years in the 173 mutation-negative non-familial SPM patients.

After correcting for melanoma type (familial or non-familial), number of primaries (single or multiple), gender and disease occurrence (incident or prevalent), we found that the effect of mutation on the age at diagnosis was significant ($P = 0.012$).

Families of mutation-positive non-familial SPM patients

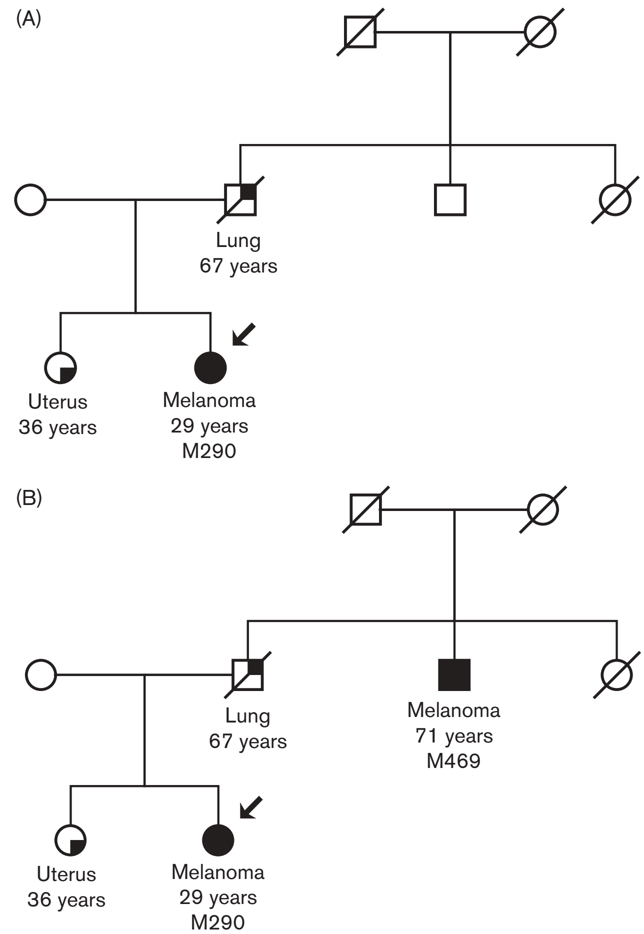
To date, the families of two of the 17 CDKN2A mutation-positive non-familial SPM patients (probands M290 and M345) have agreed to be enrolled in the second phase of the study. Prior to enrolment, none of these individuals had undergone skin examinations for a number of years.

Table 2 **CDKN2A mutations and age at onset in familial and non-familial SPM and MPM patients**

Melanoma group	Mutation-negative	Mutation-positive	Total
Familial			
n (cases)	25	9	34
Mean (SD)	46 (14.9)	38 (12.0)	44 (14.4)
Median	47	41	45.5
Non-familial SPM			
n (cases)	173	17	190
Mean (SD)	48 (16.7)	39 (15.7)	47 (17.0)
Median	46	32	45
Non-familial MPM			
n (cases)	10	4	14
Mean (SD)	50 (16.2)	46 (14.2)	49 (15.6)
Median	54.5	48	51.5

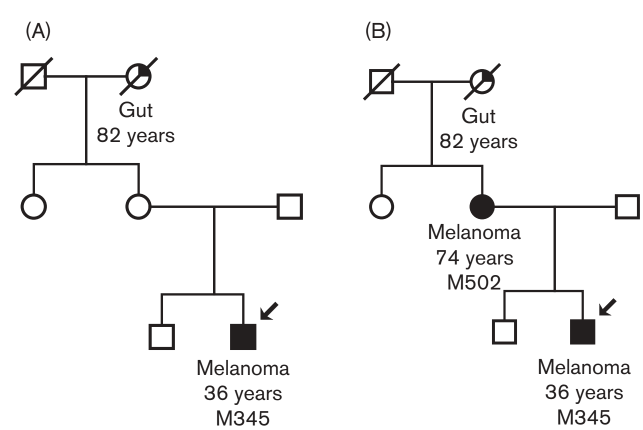
MPM, multiple primary melanoma; SD, standard deviation; SPM, single primary melanoma.

Fig. 1



Family history of individual M290, carrier of the G101W mutation in the CDKN2A gene, before (A) and after (B) genetic counselling and detailed dermatological examination. Arrows indicate the proband.

Fig. 2



Family history of individual M345, carrier of the G101W mutation in the CDKN2A gene, before (A) and after (B) genetic counselling and detailed dermatological examination. Arrows indicate the proband.

Table 3 Haplotype analysis for 9p markers in G101W-positive probands. The region of genotype sharing is indicated in bold. Genotypes in bold are consistent with the shared haplotypes previously reported in Ciotti *et al.* [4] (a) SPM G101W-positive probands

	SPM G101W cases													
Marker	M14	M61	M159	M296	M323	M338	M350	M360	M386	M488	M499	M523	M559	M588
IFNA	3 **4**	3 3	5 **4**	3 **4**	3 **4**	2 **4**	3 **4**	3 2	2 3	3 **4**	2 **4**	3 **4**	3 **4**	2 **4**
D9S736	3 **3**	4 **3**	4 **3**	3 **3**	6 **3**	5 **3**	4 **3**	4 4	3 **3**	3 **3**	3 **3**	4 **3**	3 **3**	4 **3**
D9S1749	15 **20**	30 18	24 **20**	24 22	13 19	25 **20**	26 21	25 10	19 17	22 17	32 **20**	24 19	20 **20**	21 **20**
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D9S974	5 **6**	6 **6**	8 **6**	4 **6**	8 **6**	3 **6**	3 **6**	8 **6**	6 **6**	6 **6**	2 7	3 **6**	3 **6**	7 **6**
D9S942	9 **9**	5 **9**	23 **9**	4 16	17 **9**	19 **9**	19 **9**	17 **9**	12 **9**	4 **9**	19 **9**	19 **9**	19 **9**	9 **9**
D9S1748	8 **9**	14 **9**	6 **9**	9 **9**	7 **9**	8 **9**	8 **9**	3 **9**	9 **9**	5 **9**	8 **9**	10 7	8 **9**	8 **9**
D9S1604	2 **2**	1 **2**	1 **2**	1 **2**	2 **2**	1 **2**	1 **2**	2 **2**	2 **2**	2 **2**	2 **2**	1 **2**	1 **2**	2 **2**
D9S171	5 **5**	6 8	10 **5**	1 **5**	4 **5**	10 **5**	8 **5**	5 **5**	1 **5**	10 **5**	1 **5**	2 10	6 10	5 **5**

Line between D9S1749 and D9S974 represents the location of the G101W mutation. SPM, single primary melanoma.

(b) MPM G101W-positive probands

	MPM G101W cases		
Marker	M458	M594	M250
IFNA	6 **4**	3 **4**	2 **4**
D9S736	3 **3**	4 **3**	4 **3**
D9S1749	21 15	28 24	21 21
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D9S974	8 **6**	8 **6**	12 **6**
D9S942	17 **9**	17 **9**	13 **9**
D9S1748	7 **9**	7 **9**	7 **9**
D9S1604	2 **2**	2 **2**	2 **2**
D9S171	10 **5**	8 10	1 **5**

Line between D9S1749 and D9S974 represents the location of the G101W mutation. MPM, multiple primary melanoma.

The mother and paternal uncle of proband M290 were found to be wild-type and G101W carriers, respectively. The mother of proband M345 was found to be G101W mutation-positive. Detailed dermatological examination of the two mutation-positive relatives revealed that they had *in situ* and thin lesions (0.3 mm) at 71 years and 74 years, respectively (see pedigrees of individuals with mutations in Figs 1 and 2).

Haplotype analysis

Analysis of markers flanking the CDKN2A locus showed that genotypes of G101W carriers were consistent with the previously reported founder G101W core haplotype [4]; however, the phase could not be determined for most patients (Table 3; data not shown for the nine G101W-positive hospital-based familial patients). As expected, the genotypes for D74Y and P48T were not consistent with the G101W core haplotype (data not shown). Phase was determined only for the two G101W probands (M290 and M345) and their mutation-positive relatives. Both probands carried the G101W core haplotype (Fig. 3).

Discussion

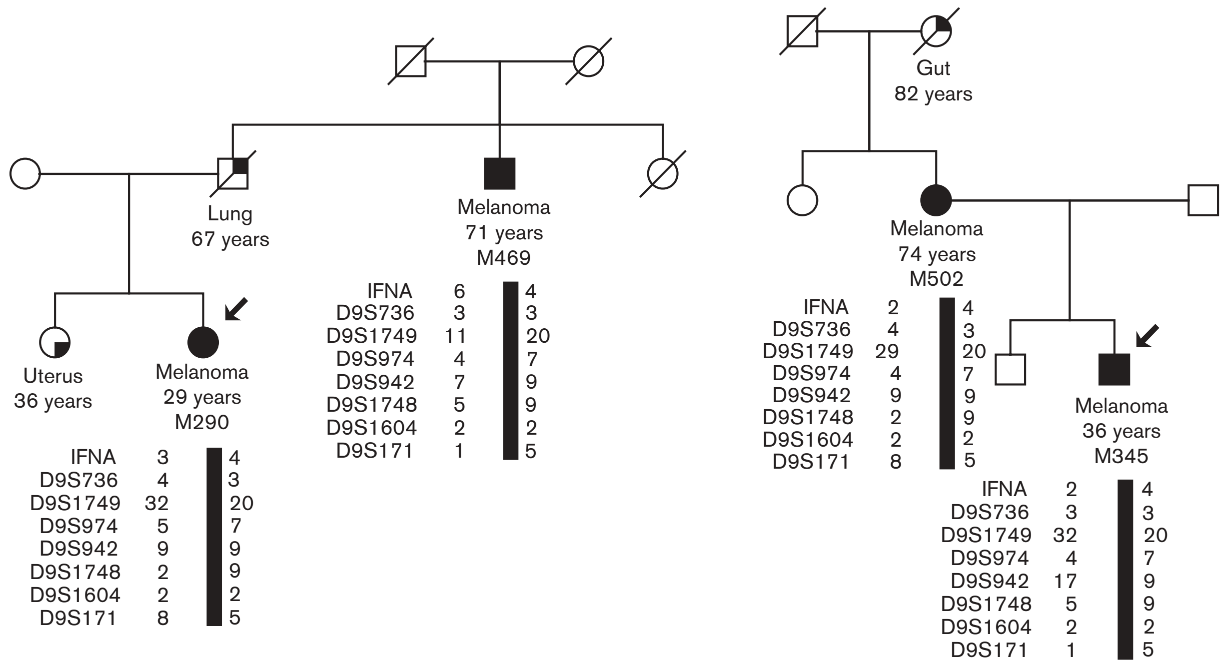
Although the presence of multiple cases of melanoma on the same side of a kindred is acknowledged to be the best predictor of CDKN2A mutation, we have previously found that melanoma families in Liguria display a high frequency of the G101W founder mutation despite their low density [3]. Therefore, the need has arisen to identify other markers of heritable susceptibility in our region. For this purpose, we have investigated the hypothesis that an early age at onset (possibly at ≤ 40 years) could be a good predictor through the observation of a hospital-based series of Ligurian melanoma patients. To date, no other study has specifically focused on the frequency of mutation amongst non-familial SPM cases in a region with a prevalent founder mutation, but a similar analysis has been conducted with regard to BRCA1 and BRCA2 genes in Ashkenazi carriers [12].

Screening of hospital-based patients revealed that the G101W mutation frequency was high amongst familial (26%) and non-familial MPM (21%) cases, as expected, and that roughly 8% of the 190 non-familial SPM cases were G101W-positive, whilst one patient carried a novel single base pair substitution, D74Y. To our knowledge, the D74Y mutation has not been reported elsewhere; it occurs at a site which is critical to function as predicted by the ankyrin structure of the p16 protein [13].

Having found that CDKN2A mutation-positive cases have a significantly earlier age at diagnosis than those carrying no mutation in that gene ($P = 0.012$), after correcting for melanoma type (familial or non-familial), number of primaries (single or multiple), gender and disease occurrence (incident or prevalent), it can be inferred that, in Liguria, an early age at diagnosis could be predictive of positive mutation status irrespective of family history.

Our findings are not dissimilar to those reported for MPM in other areas with a predominant founder mutation [7]. Auroy *et al.* [14] reported similar results for MPM cases carrying the same G101W founder mutation identified in our Ligurian patients, as confirmed by haplotype analysis [4].

Fig. 3



Haplotype analysis for 9p markers in two single primary melanoma (SPM) G101W-positive probands (M290 and M345) and their relatives (M469 and M502). The region of genotype sharing is indicated by a black bar. Genotypes delineated are consistent with the shared haplotypes previously reported in Ciotti *et al.* [4]. Segregating alleles in M290 and M345 could not be unequivocally determined for markers IFNA and D9S974.

Conversely, our data are in contrast with the only previous study on the frequency of CDKN2A mutations in early onset patients [8]. The authors reported a very low incidence of mutations in young melanoma patients from Massachusetts, where no founder effect has been identified. Given the lower incidence of melanoma in Italy [9], although it is a sunny area, compared with the USA [8], it is likely that, in our region, a younger age at diagnosis reflects the impact of genetic factors rather than high sun exposure, as the latter could result in early age at diagnosis in both carriers and non-carriers.

It should be noted that the sample of patients who agreed to participate in our study showed a significantly earlier age at diagnosis and included a higher number of familial cases when compared with the set of subjects who either declined or were not asked. These differences may be explained by the greater willingness, in our experience, of younger individuals to participate in genetic studies, and by the fact that patients who have affected relatives are more likely to perceive that they are at risk of developing the disease [15].

We conclude that DNA testing of early onset melanoma patients (for example, cases diagnosed at ≤ 40 years) may be warranted in Liguria, and indeed may help to identify new, at-risk kindreds that could be usefully targeted for prevention strategies, as supported by early findings in the second phase of our study. However, before we can precisely define the age groups to be targeted, our results need to be confirmed by larger, population-based studies. Furthermore, because UV exposure in early life appears to increase the risk of melanoma in adulthood in low-risk families, further studies should include data on lifetime sun exposure [16]. Such analyses could explain whether the history of sun exposure of all genotyped patients in Liguria significantly affects the age at onset of melanoma [17].

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